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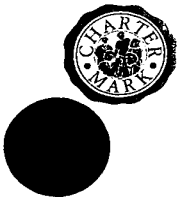

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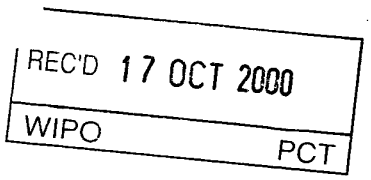

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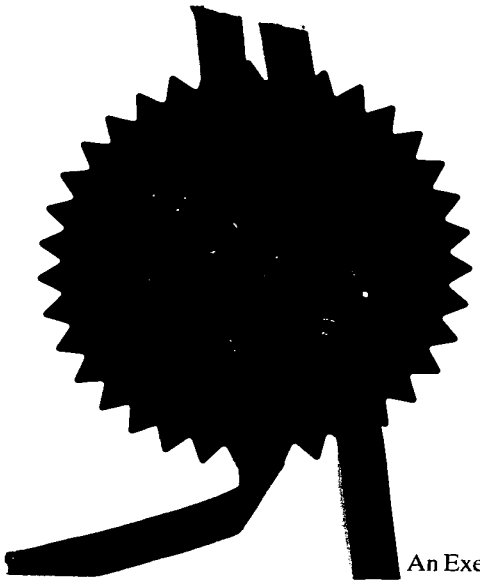

Signed

Dated 02 OCT 2000

GB9922527.8

By virtue of a direction given under Section of the Patents Act 1977, the application is proceeding in the name of

ASTRAZENECA AB,
Incorporated in Sweden,
S-151 85 Sodertalje,
Sweden

[ADP No. 07822448003]

GB9922527.8

By virtue of a direction given under Section of the Patents Act 1977, the application is proceeding in the name of

ASTRAZENECA UK LIMITED
Incorporated in the United Kingdom
15 Stanhope Gate
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W1Y 6LN
United Kingdom

[ADP No. 07810294001]

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1. Your reference — PHM.99-139/GB/P

2. Patent application number *(The Patent Office will fill in this part)* — **9922527.8** — 24 SEP 1999

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Zeneca Limited
15 Stanhope Gate
London. W1Y 6LN
GB.

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Patents ADP number *(if you know it)* 6254007002

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention — ASSAY

5. Name of your agent *(if you have one)* — PHILLIPS, Neil Godfrey Alasdair

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ASSAY

This invention relates to a diagnostic method for the simultaneous detection of the variable length polythymidine (polyT) tract alleles in the CFTR gene that are associated with the phenotypic modulation of selected CFTR mutations (Friedman KJ. et al. Hum Mutat. 10: 108-115 (1997)).

The invention also relates to a diagnostic method further comprising the simultaneous detection of one or more of the DF508 (Kerem B. et al. Science 245: 1073-1080 (1989)), 621+1G>T (Rozen R. et al. Am. J. Med. Genet. 42: 360-364 (1992)), G542X, 3659delC, A455E, DI507 (Kerem B. et al. Proc. Nat.Acad. Sci. 87: 8447-8451 (1990)), 3849+10kbC>T (Highsmith WE. et al. New Eng. J. Med. 331: 974-980 (1994)), N1303K (Osborne L. et al. Am. J. Hum. Genet. 48: 608-612 (1991)), 1717-1G>A (Kerem B. et al. Proc. Nat. Acad. Sci. 87: 8447-8451 (1990), Guillermit H. et al. Hum. Genet. 85: 450-453 (1990)), 1078delT (Claustres M. et al. Genomics 13: 907-908 (1992)), W1282X (Vidaud M et al. Hum. Genet. 85: 446-449 (1990)), R347P, R117H (Dean M. et al. Cell 61: 863-870 (1990)), G551D, R553X (Cutting GR. et al. New Eng. J. Med. 323: 1685-1689 (1990)), S1251N (Gasparini P. et al. Hum. Mutat. 2: 389-394 (1993)), R1162X, R334W (Gasparini P. et al. Genomics 10: 193-200 (1991)), 2183AA>G, (Bozon D. et al. Hum. Mutat. 3: 330-332 (1994)), and the E60X (Will K. et al. Hum. Mutat. 5: 210-20 (1995)) mutations in the human cystic fibrosis conductance regulator (CFTR) gene that are prevalent in populations of European descent using the amplification refractory mutation system, (ARMS).

The invention also relates to a diagnostic method further comprising the simultaneous detection of one or more of the G85E (Chalkley G. et al. J. Med. Genet. 28: 875-877 (1991)), 405+1G>A (Dork T. et al. Hum Mol Genet. 2: 1965-1966 1993)), S549R (Kerem B. et al. Proc. Nat.Acad. Sci. 87: 8447-8451 (1990)), (or, depending on which of the two mutations produce this phenotype is tested for, Sangiuolo. et al. Genomics 9: 788-789 (1991)), W1089X (Shoshani T. et al. Hum. Molec. Genet. 3: 657-658 (1994)), D1152H (Feldmann D. et al. Clin. Chem. 41: 1675 (1995)) mutations in the human CFTR gene that are prevalent in populations of Middle Eastern descent using ARMS.

The invention also relates to mutation specific and allele specific primers for use in the method, to diagnostic kits containing these primers and to techniques for detecting primer specific amplification products.

Cystic fibrosis transmembrane conductance regulator (CFTR) functions as a chloride channel and controls the regulation of other transport pathways. The CFTR gene maps to chromosome 7q (Riordan J R. et al. Science 245: 1066-1073 (1989)). Mutations in the CFTR gene cause cystic fibrosis (CF) formerly known as cystic fibrosis of the pancreas. Cystic fibrosis is an autosomal recessive disorder mainly among Caucasians with a frequency of approximately 1/2 500 (Welsh MJ. et al. in Scriver et al. (eds): The Metabolic Basis of Inherited Disease, Vol 3: 7th ed. McGraw-Hill; New York, pp3799-3876 (1995)). Cystic fibrosis disrupts exocrine function of the pancreas, intestinal glands (meconium ileus), biliary tree (biliary cirrhosis), bronchial glands (chronic bronchopulmonary infection with emphysema), and sweat glands (high sweat electrolyte with depletion in a hot environment). Infertility occurs in both males and females. Many of the clinical and pathological findings are thought to be attributable to a generalised defect in mucus secretion due to an abnormality in the chloride channel.

The treatment of CF is complex, costly and time-consuming. Most children with CF in the developed world are followed in CF clinics which are typically multidisciplinary, involving physicians, nurses, social workers, nutritionists and physiotherapists. Treatment is directed at improving nutrition through the use of replacement pancreatic enzymes and vitamins, as well as a high-energy, high-protein and liberal-fat diet. For those not responding to this approach, enteral supplementation by nightly nasogastric, gastrostomy or jejunostomy infusion of high-energy diets has been used. Pulmonary treatment includes antibiotic therapy, either maintained continuously or reserved for exacerbations, and chest physiotherapy consisting of postural drainage, percussion, vibration and assisted coughing.

Approximately 70% of the mutations in CF patients correspond to a specific deletion of 3 basepairs, which results in the loss of a phenylalanine residue at amino acid position 508 of the CFTR protein (Kerem B. et al. Am. J. Hum. Genet. 44: 827-834 (1989)).

Mutations of the CFTR gene are believed to produce an abnormal protein as a component of the chloride channel gate at the cell surface and are classified according to their phenotypic manifestations. Class I are nonsense mutations, resulting in the introduction of a stop codon, and in no synthesis of CFTR e.g. G542X, R553X, W1282X. Class II mutations cause a block in CFTR assembly in the endoplasmic reticulum or affect transport to the cell membrane, these include ΔF508, A455E, and P574H. Class III and IV mutations give rise to defective chloride channel activity or regulation e.g. R117H, which alters amino acid residues

in the first CFTR transmembrane domain. Class V mutations result in modulations in CFTR synthesis. In cases of CF involving class I and II CFTR mutations (most commonly ΔF508 homozygotes) chronic pulmonary disease is observed at variable levels with chronic pancreatitis. However, some CF patients present with "mild" symptoms ranging from sinopulmonary problems with pancreas sufficiency (PS-CF), to congenital bilateral absence of vas deferens (CBAVD). Mutations associated with PS-CF include R334W and R117H; the latter mutation accounts for approximately 0.8% of mutant alleles in Caucasian CF patients (Tsui LC. Trends Genet. 57: 392-398 (1992)).

There is a highly conserved polyT at the end of intron 8 of the CFTR gene. This polyT tract plays an important role in aiding splice branch site recognition, and identifying and using the splice acceptor site (Smith CW. et al. Nature. 342: 243-247 (1989)). There are three allelic variants depending on the number of thymidines (5, 7, or 9) with allele frequencies in the general population of 5%, 85%, 10%, and respectively (Chu C-S. et al. EMBO J. 10: 1355-1363 (1991)). The number of thymidines determines the efficiency by which the intron 8 splice acceptor site is used. The efficiency decreases when a shorter stretch of thymidine residues is found. The T5 allele results in the most inefficient use of this splice acceptor site (Teng, H. et al. Hum. Mol. Genet. 6: 85-90 (1997)) and so CFTR transcripts from a T5 allele will lack exon 9 sequence. Individuals homozygous for the 5T allele were found to generate 90% CFTR mRNA with exon9 skipped (exon9$^-$). While 7T homozygotes generated <25% exon9$^-$ transcripts, and 9T <15% (Lissens W. et al. Hum. Reprod. 11: Supp4 55-77 (1996)).

There is an association between phenotype and the particular polyT allelic background for some CF mutations, for example, if a CFTR gene with the R117H mutation harbors a T5 allele, the mutant gene will be responsible for CF. An R117H mutant CFTR gene that harbors a T7 allele can either result in CF or CBAVD (Kiesewetter S. et al. Nature Genet. 5: 274-278, (1993)).

The existence of polyT splice site variants may therefore explain the clinical heterogeneity observed in some mild cases of CF. It is also possible that the CF clinical phenotypes associated with mutations other than ΔF508 result from both the mutation in the CFTR gene and the sequence at exon9 splice site acceptor site.

Most CFTR mutations occur on a particular polyT background, most commonly the 9T allele; ΔF508 is in linkage disequilibrium with the 9T allele and the mild CF mutation R117H is always found on a 5T or 7T background. The mutation R117H has been shown to

owe its phenotypic heterogeneity (PS CF or CBAVD) to the presence of the 5T or 7T polyT alleles (Friedman KJ. et al. Hum Mutat. 10: 108-115 (1997)). Compound heterozygotes having the genotype ΔF508/R117H may present as PS-CF, CBAVD or be asymptomatic. Since the mutation ΔF508 is in linkage disequilibrium with the 9T allele, it has been demonstrated that the R117H phenotype contrasts with respect to its associated polyT haplotype (R117H-5T or R117-7T). The incidence of 5T alleles is also associated with other clinical phenotypes, for example, disseminated bronchiectasis (Pignatti PF. et al. Am. J. Hum. Genet. 58: 889-892 (1996)). Hence the human CFTR gene intron 8 5T, 7T and 9T allele discriminatory test described and disclosed here is a test that is particularly useful when used in combination with any other cystic fibrosis diagnostic test.

Several cohort studies of screened and unscreened subjects have suggested that people identified with CF in the presymptomatic phase do better than those in whom a diagnosis is made because of symptoms. For example, one study (Dankert-Roelse JE. et al. J. Pediatr. 114: 362-367 (1989)) showed that, 88% of screened children but only 60% of unscreened children were still alive at age 11 years.

Diagnostic techniques for phenotypic manifestations of CF include measuring sweat chloride. Elevated sweat chloride concentrations (greater than 60 mmol/l) are almost exclusively observed in patients with CF. However, it is difficult to obtain sufficient sweat from newborns, even after stimulating localised sweating by administering pilocarpine into the skin it is often impossible to collect sufficient sweat for accurate analysis. Sweat chloride concentrations can be measured, for example, by using the Lazar ISM-146 Micro Chloride electrode. Measurement of elevated immunoreactive trypsinogen (IRT) in a dried blood spot is another screening method for CF. False positives and false negatives are known to occur, with false negatives occurring more frequently in neonates with meconium ileus. The positive predictive value of the test is only 1-7% (Ryley HC. J. Clin. Pathol. 41: 726-729 (1988), Edminson PD. et al. Scand. J. Gastroenterol. Suppl. 143: 13-18 (1988)). A false positive rate of 93-99% could and likely does generate considerable anxiety, which may be long-lasting (Ryley HC. J. Clin. Pathol. 41: 726-729 (1988)). Furthermore, elevations of IRT decline after the first few months of life, so while exact timing of specimen collection in the neonatal period is not critical, the collection of a second screening specimen to follow-up an initial abnormal screen should occur no earlier than 21 days, to avoid an increased number of false positives, and no later than 60 days, to reduce the risk of false negatives. Some newborn

screening programs rely on other tests. The "BM meconium test" identifies infants with high albumin content in the stool resulting from pancreatic insufficiency but it has very low sensitivity (Naylor EW. Semin. Perinatol. 9: 232-249 (1985)).

Friedman et al. describe an allele-specific amplification polyT assay (Friedman KJ. et al. Hum. Mutat. 10: 108-115 (1997)) designed to screen individuals for CFTR intron 8 5T, 7T, 9T alleles and heterozygotic polyT allelic combinations. However, the assay described by Friedman requires the use of each allele-specific primer in a separate reaction.

In our European Patent No. 0 332 435 B1 we disclose the Amplification Refractory Mutation System (ARMS). This simple and elegant method permits the detection of point mutations via allele-specific amplification of target sequences. In EP-0 332 435 we disclose and claim the application of ARMS to a variety of inherited and/or acquired genetic disorders.

Multiplex allele-specific amplification assays have been described for the simultaneous analysis of several CF causing mutations (for example, see Ferrie RM. et al. Am. J. Hum. Genet. 51: 251-262 (1992) and Robertson NH. et al. Eur. Respir. J. 12: 477-482 (1998)) as disclosed in our European Patent No. 0 928 832. The combination of different primers for the simultaneous detection of two or more point mutations is termed "multiplexing". However, the design of robust and accurate allele-specific multiplex tests is not straightforward. A person skilled in the art would still need to contend with inter- and intra- primer reactions when several alleles are amplified simultaneously in one reaction; there may be further interactions between amplification products for one allele with the primers for another allele. Furthermore, there will be inequalities between the efficiencies amplification products from different alleles.

We now provide allele-specific primers for the 5T, 7T and 9T alleles in intron 8 of the CFTR gene that are designed for use in combination in a multiplex assay and function by bringing about an allele-specific size differential between CFTR intron 8 5T, 7T and 9T derived amplification products.

Therefore according to a first aspect of the invention we now provide a diagnostic method for the detection of the 5T, 7T and 9T alleles in intron 8 of the human CFTR gene which method comprises contacting a test sample of nucleic acid from an individual with a multiplex of diagnostic primers comprising (i) 5T variant primer 5'(N)nAAAGAC3', (ii) 7T variant primer 5'(N*)n*(N)nAAAAGC3' and (iii) 9T variant primer

5'(N*)n*(N)nAAAATC3', wherein N represents additional nucleotides which base pair with the corresponding genomic sequence in the respective allele and n is an integer between 10 and 30 and N* represents additional non-homologous nucleotides which do not base pair with the corresponding genomic sequence in the respective allele and n* is an integer between 5 and 60, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that a diagnostic primer is extended only when the corresponding allelic variant is present in the sample; and detecting the presence or absence of the allelic variant by reference to the presence or absence of a diagnostic primer extension product.

It will be appreciated that N* may be any other molecule that serves to reduce the electrophoretic mobility of an extension product such as 3'hexaethylene glycol (HEG) or a combination of such molecules and additional non-homologous nucleotides.

The diagnostic primers for use in the methods of the invention conveniently comprise one or more of

5T variant primer 5'(N)nTGTTAAAGAC3',

7T variant primer 5'(N*)n*(N)nTTAAAAAAGC3' and

9T variant primer 5'(N*)n*(N)nAAAAAAAATC3' wherein N, n, N* and n* are as defined above and n is an integer between 6 and 26.

It will be understood that the nucleotide sequence as defined by (N)n in the diagnostic primer is normally selected to be 100% complementary to the corresponding genomic sequence. However, as required, one or more mismatched bases may be included, for example at the 5' terminus of the primer. For example up to two, three, four or five mismatched base pairs may be included in the nucleotide sequence defined by (N)n. It will also be understood that any mismatched bases must not significantly impair the discriminatory properties of the diagnostic primer.

The integer n is for example 10, up to 15, up to 20, up to 25, or up to 30.

Preferred diagnostic primers include

5T variant primer 5'TAATTCCCCAAATCCCTGTTAAAGAC3',

7T variant primer 5'(N*)n*TAATTCCCCAAATCCCTGTTAAAAAAGC3' and

9T variant primer 5'(N*)n*TAATTCCCCAAATCCCTGTTAAAAAAAATC3' wherein N* and n* are as defined above.

Preferred diagnostic 7T and 9T primers are: 7T variant primer

5'GTTAATCATTCAGCTACTACGCACCTAATTCCCCAAATCCCTGTTAAAAAAGC3'

and 9T variant primer
5'GACTGTACGATACTCATTTATATGAAGTCAGCTACTTACCTATAGAACGCTTGC
TAGTTTAATTCCCCAAATCCCTGTTAAAAAAAATC3'.

The above primers have been shown to detect their respective alleles reliably and robustly. Each of the primers disclosed above represents a further and independent aspect of the invention.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer in one or more cycles of PCR amplification. A convenient example of this aspect is set out in our European patent number EP-B1-0332435. Any convenient amplification primer may be useed provided that the resulting amplification products are of a suitable size for separation and analysis. The further amplification primer is conveniently the polyT common primer GTACATAAAACAAGCATCTATTGAAAATATCTGAC and this is used in combination with the intron 8 5T, 7T and 9T allele-specific primers.

Therefore according to a further aspect of the invention we provide a diagnostic method for the detection of the 5T, 7T and 9T alleles in intron 8 of the human CFTR gene which method comprises contacting a test sample of nucleic acid from an individual with a multiplex of diagnostic primers comprising (i) 5T variant primer 5'(N)nAAAGAC3', (ii) 7T variant primer 5'(N*)n*(N)nAAAAGC3' and (iii) 9T variant primer 5'(N*)n*(N)nAAAATC3', wherein N represents additional nucleotides which base pair with the corresponding genomic sequence in the respective allele and n is an integer between 10 and 30 and N* represents additional non-homologous nucleotides which do not base pair with the corresponding genomic sequence in the respective allele and n* is an integer between 5 and 60, and a common amplification primer in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, and subjecting the mixture to PCR amplification such that a diagnostic primer is extended only when the corresponding allelic variant is present in the sample; and detecting the presence or absence of the allelic variant by reference to the presence or absence of a corresponding PCR amplification product.

The above aspects of the invention are referred to as primer set 1 and conveniently illustrated by reference to Table 1 and the disclosure of a specific primer mix1.

References to primer "mixes" and "sets" are not intended to be limiting and the terms are used throughout the text interchangeably.

It will also be appreciated that we do not want to be limited solely to discrimination between the human CFTR gene intron 8 5T, 7T and 9T alleles by allele associated PCR product sizes; hence a further definition of N* is one from a group of labels that can be conjugated to primers whereby an individual label is associated specifically to one allele-specific primer and n* may be 0 or an integer greater than 1. An example of labels according to this definition of N* is a group of molecules such as fluorophores for example, (Brown T. and Brown DJS. in Newton CR. (ed): PCR - Essential Data: 1st ed. Wiley; Chichester, pp57-71 (1995)).

The primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example *"Protocols For Oligonucleotides And Analogues: Synthesis And Properties;"* Methods In Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition.

It will be appreciated that any of the above diagnostic methods may, if appropriate, also be configured so that extension of the diagnostic primer indicates the absence of the respective CFTR gene polyT allele.

As outlined earlier above, the polyT methods of this invention may be used with any known CFTR testing procedure.

In addition we have now devised novel diagnostic primer sequences for the detection of the W1282X, 1717-1G>A, G542X, N1303K, DF508 and 3849+10kbC>T mutations of the human CFTR gene using ARMS allele specific amplification. Therefore the polyT multiplex of the present invention is conveniently accompanied by the use in a separate ARMS reaction of one or more of :

DF508 mutant primer 5'(N)nACCATT3',

3849+10kb C>T mutant primer 5'(N)nTACGCA3',

N1303K mutant primer 5'(N)nTCCATC3',

1717-1G>A mutant primer 5'(N)nTAATTA3',

W1282X mutant primer 5'(N)nCAGTCA3', and

G542X mutant primer 5'(N)nTTCTCT3' wherein N and n are as previously defined, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that a diagnostic primer is extended only when the corresponding allelic variant is present in the sample; and detecting the presence or absence of the allelic variant by reference to the presence or absence of a diagnostic primer extension product.

Two or more of the above diagnostic primers are conveniently used as a multiplex and more conveniently with a suitable amplificiation primer. Preferably several or all of the above primer sequences are used in a single multiplex reaction.

Convenient primers include:

W1282X mutant primer 5'(N)nGCAACAGTCA3',

1717-1G>A mutant primer 5'(N)nTTGGTAATTA3',

G542X mutant primer 5'(N)nATAGTTCTCT3',

N1303K mutant primer 5'(N)nGGGATCCATC3',

DF508 mutant primer 5'(N)nAAACACCATT3' and

3849+10kb C>T mutant primer 5'(N)nGTCTTACGCA3' wherein N, n, are as defined above and n is an integer between 6 and 26.

Preferred diagnostic primers include

W1282X mutant primer 5'TCTTGGGATTCAATAACTTTGCAACAGTCA3',

1717-1G>A mutant primer 5'TCTCGAATTTTCTATTTTTGGTAATTA3',

G542X mutant primer 5'AGTTTGCAGAGAAAGACAATATAGTTCTCT3',

N1303K mutant primer 5'TGATCACTCCACTGTTCATAGGGATCCATC3',

DF508 mutant primer 5'GTATCTATATTCATCATAGGAAACACCATT3', and

3849+10kb C>T mutant primer 5'GAACATTTCCTTTCAGGGTGTCTTACGCA3'.

The above aspects of the invention are referred to as primer set 2A and illustrated by reference to Table 3 and the specific primer mix 2A. Primer mix 2A is conveniently used in combination with primer mix 1 as set out in Table 1.

We have also devised novel diagnostic primer sequences for detection by ARMS allele specific amplification of the DF508 normal allele, and the 621+1G>T, R117H, R334W, G551D, R553X, and R1162X mutations of the human CFTR gene. Therefore the polyT multiplex of the present invention is conveniently accompanied by the use in a separate ARMS reaction of one or more of :

DF508 non-mutant primer 5'(N)nACCACA3',

W1282X mutant primer 5'(N)nCAGTCA3',

1717-1 mutant primer 5'(N)nTAATTA3',

G542X mutant primer 5'(N)nTTCTCT3',

N1303K mutant primer 5'(N)nTCCATC3',

DF508 non-mutant primer 5'(N)nACCACA3',

DF508 mutant primer 5'(N)nACCATT3' and

3849+10kb C>T mutant primer 5'(N)nTACGCA3' wherein N and n are as previously defined, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that a diagnostic primer is extended only when the corresponding allelic variant is present in the sample; and detecting the presence or absence of the allelic variant by reference to the presence or absence of a diagnostic primer extension product.

Two or more of the above diagnostic primers are conveniently used as a multiplex and more conveniently with a suitable amplificiation primer. Preferably several or all of the above primer sequences are used in a single multiplex reaction.

Convenient diagnostic primers include:

DF508 non-mutant primer 5'(N)nAAACACCACA3',

R117H mutant primer 5'(N)nGCGATAGACT3',

621+1G>T mutant primer 5'(N)nGAAGTATTGA3',

R334W mutant primer 5'(N)nATCATCCTGT3',

R1162X mutant primer 5'(N)nTCTGTGAGTT3',

R553X mutant primer 5'(N)nTTCTTGCTGA3' and

G551D mutant primer 5'(N)nGCTCGTTGTT3' wherein N, n, are as defined above and n is an integer between 6 and 26.

Preferred diagnostic primers include:

R117H mutant primer5'AGCCTATGCCTAGATAAATCGCGATAGACT3',

621+1G>T mutant primer 5'TGCCATGGGGCCTGTGCAAGGAAGTATTGA3',

R334W mutant primer 5'CCTATGCACTAATCAAAGGAATCATCCTGT3',

R1162X mutant primer 5'TATTTTTATTTCAGATGCGATCTGTGAGTT3',

R553X mutant primer 5'TTATTCACCTTGCTAAAGAAATTCTTGCTGA3',

G551D mutant primer 5'GCTAAAGAAATTCTTGCTCGTTGTT3'.

The above aspects of the invention are referred to as primer set 2B and are conviently illustrated by reference to Table 3 and the specific primer mix 2B. Primer mix 2B is conveniently used in combination with mix1 as set out in Table 1.We have also devised novel diagnostic primer sequences for the A455E, 2183AA>G, 3659delC, DI507, 1078delT, R347P, S1251N and E60X mutations of the human CFTR gene using ARMS allele specific amplification. Therefore the polyT multiplex of the present invention is conveniently accompanied by the use in a separate ARMS reaction of one or more of :

A455E mutant primer 5'(N)nGTTGTA3',

2183AA>G mutant primer 5'(N)nGATAGC3',

3659delC mutant primer 5'(N)nCCTAGA3',

DI507 mutant primer 5'(N)nATAACT3',

1078delT mutant primer 5'(N)nTTCCTG3',

R347P mutant primer 5'(N)nTCTACC3',

S1251N mutant primer 5'(N)nGAAGCA3' and

E60X mutant primer 5'(N)nCAGTTA3'

wherein N and n are as previously defined, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that a diagnostic primer is extended only when the corresponding allelic variant is present in the sample; and detecting the presence or absence of the allelic variant by reference to the presence or absence of a diagnostic primer extension product.

Two or more of the above diagnostic primers are conveniently used as a multiplex and more conveniently with a suitable amplificiation primer. Preferably several or all of the above primer sequences are used in a single multiplex reaction.

The diagnostic primer conveniently comprises one or more of

A455E mutant primer 5'(N)nAGTTGTTGTA3',

1078delT mutant primer 5'(N)nAGGGTTCCTG3',

R347P mutant primer 5'(N)nTTGTTCTACC3',

DI507 mutant primer 5'(N)nGAAAATAACT3',

3659delC mutant primer 5'(N)nTAAACCTAGA3',

2183AA>G mutant primer 5'(N)nAAAAGATAGC3',

S1251N mutant primer 5'(N)nCAGGGAAGCA3' and

E60X mutant primer 5'(N)nAAGCCAGTTA3' wherein N, n, are as defined above and n is an integer between 6 and 26.

Preferred diagnostic primers include

A455E mutant primer 5'TTCAAGATAGAAAGAGGACAGTTGTTGTA3',

1078delT mutant primer 5'CCTTCTTCTTCTCAGGGTTCCTG3',

R347P mutant primer 5'CACCATCTCATTCTGCATTGTTCTACC3',

DI507 mutant primer 5'GCCTGGCACCATTAAAGAAAATAACT3',

3659delC mutant primer 5'ATGCCAACAGAAGGTAAACCTAGA3',

2183AA>G mutant primer 5'CAAACTCTCCAGTCTGTTTAAAAGATAGC3',
S1251N mutant primer 5'GGAAGAACTGGATCAGGGAAGCA3' and
E60X mutant primer 5'TTAGGATTTTTCTTTGAAGCCAGTTA3'.

The above aspects of the invention are referred to as primer set 2C and conveniently illustrated by reference to Table 4 and the specific primer mix 2C. Primer mix 2C is conveniently used in combination with mix1 as set out in Table 1.

We have also devised novel diagnostic primer sequences for the detection of the G85E, 405+1G>A, S549R, W1089X and D1152H mutations of the human CFTR gene using ARMS allele specific amplification. Therefore the polyT multiplex of the present invention is conveniently accompanied by the use in a separate ARMS reaction of one or more of :

G85E mutant primer 5'(N)nCTACGA3',
405+1G>A mutant primer 5'(N)nTAGTGA3',
S549R mutant primer 5'(N)nCTGACG3',
W1089X mutant primer 5'(N)nCAAATA3' and
D1152H mutant primer 5'(N)nCACTTG3' wherein N and n are as previously defined, in the presence of appropriate nucleotide triphosphates and an agent for polymerisation, such that a diagnostic primer is extended only when the corresponding allelic variant is present in the sample; and detecting the presence or absence of the allelic variant by reference to the presence or absence of a diagnostic primer extension product.

Two or more of the above diagnostic primers are conveniently used as a multiplex and more conveniently with a suitable amplificiation primer. Preferably several or all of the above primer sequences are used in a single multiplex reaction.

The diagnostic primer conveniently comprises one or more of
G85E mutant primer 5'(N)nTGTTCTACGA3',
405+1G>A mutant primer 5'(N)nTATTTAGTGA3',
S549R mutant primer 5'(N)nCACACTGACG3',
W1089X mutant primer 5'(N)nCTGCCAAATA3',
D1152H mutant primer 5'(N)nTATCCACTTG3' wherein N, n, are as defined above and n is an integer between 6 and 26.

Preferred diagnostic primers include:

G85E mutant primer
5'TAGCCATTGATGACGGAGCGATGTTTTTTCTGGAGATTTATGTTCTACGA3'
405+1G>A mutant primer
5'GATTTATGTTCTATGGAATCTTTTTATATTTAGTGA3',
S549R mutant primer 5'TGGAGAAGGTGGAATCACACTGACG3',
W1089X mutant primer 5'AAGCTCTGAATTTACATACTGCCAAATA3' and
D1152H mutant primer 5'AAAGATGATAAGACTTACCAAGCTATCCACTTG3'

The above aspects of the present invention are referred to as primer set 3 and conveniently illustrated by reference to Table 5 and the specific mix 3. Mix 3 may for example be used in combination with mix1 as set out in Table 1.

The above primers in mixes 2A, 2B, 2C and 3 have been shown to detect their respective alleles reliably and robustly.

The primers may be manufactured using any convenient method of synthesis. Examples of such methods may be found in standard textbooks, for example *"Protocols For Oligonucleotides And Analogues: Synthesis And Properties;"* Methods In Molecular Biology Series; Volume 20; Ed. Sudhir Agrawal, Humana ISBN: 0-89603-247-7; 1993; 1st Edition.

It will be appreciated that any of the above diagnostic methods may, if appropriate, also be configured so that extension of the diagnostic primer indicates the absence of the respective CFTR gene mutation or polyT allele.

The test sample of nucleic acid is preferably a blood sample but may also conveniently be a sample of any body fluid, or tissue obtained from an individual. The individual is any convenient mammal, preferably a human being. It will be appreciated that the test sample may equally be a nucleic acid sequence corresponding to the sequence in the test sample. That is to say that all or a part of the region in the sample nucleic acid may firstly be amplified using any convenient technique such as PCR before use in the method of the invention.

Any convenient enzyme for polymerisation may be used provided that it does not affect the ability of the DNA polymerase to discriminate between normal and mutant template sequences to any significant extent. Examples of convenient enzymes include thermostable enzymes which have no significant 3'-5' exonuclease activity, for example *Taq* DNA polymerase, particularly "Ampli Taq Gold"™ DNA polymerase (PE Applied Biosystems), Stoffel fragment, or other appropriately N-terminal deleted modifications of *Taq* or *Tth* (*Thermus thermophilus*) DNA polymerases.

Significiantly the diagnostic methods of the invention can be used in combination to enable the presence or absence of mutations or polyT alleles of the CFTR gene to be detected simultaneously (i.e. the method comprises several multiplex tests). Primer Mix 1 can be used in isolation or in combination with any other cystic fibrosis diagnostic tests. Because the multiplex polyT test using the mix 1 primer mix adds value to the information derived from other cystic fibrosis diagnostic tests it is convenient to use the mix 1 primer mix in combination with the mix 2A primer mix. Similarly, it is convenient to use the mix 1 primer mix in combination with the mix 2B primer mix. Similarly, it is convenient to use the mix 1 primer mix in combination with the mix 2C primer mix. Similarly, it is convenient to use the mix 1 primer mix in combination with the mix 3 primer mix. Similarly, it is convenient to use the mix 1 primer mix in combination with any two of the mix 2A, 2B, 2C or 3 primer mixes. Similarly, it is convenient to use the mix 1 primer mix in combination with any three of the mix 2A, 2B, 2C or 3 primer mixes. Similarly, it is convenient to use the mix 1 primer mix in combination with all of the mix 2A, 2B, 2C or 3 primer mixes.

The above test combinations give genotype information by distinguishing between individuals who are heterozygous and homozygous for either of the CFTR polyT alleles and the more common DF508 CFTR gene mutation. If desired, the tests may provide genotype information for the 1717-1G>A, G542X, W1282X, N1303K, 3849+10kbC>T, 621+1G>T, R553X, G551D, R117H, R1162X, R334W, A455E, 2183AA>G, 3659delC, 1078delT, DI507, R347P, S1251N, E60X, G85E, 405+1G>A, S549R, W1089X, and D1152H CFTR gene mutations by the inclusion of primers specific for the normal CFTR gene sequences that correspond to the respective mutated CFTR gene sequences associated with these mutations. Whilst each multiplex test may have any of the primers described above in combination the preferred diagnostic primer multiplexes are the mix 1 comprising primers for the 5T, 7T and 9T alleles. Mix 2A comprising primers for the 1717-1G>A, G542X, W1282X, N1303K, DF508 and 3849+10kbC>T mutations; mix 2B comprising primers for the 621+1G>T, R553X, G551D, R117H, R1162X and R334W mutations and the normal CFTR gene sequence corresponding to the DF508 mutation; mix 2C comprising primers for the A455E, 2183AA>G, 3659delC, 1078delT, DI507, R347P, S1251N and E60X mutations; mix 3 comprising primers for the G85E, 405+1G>A, S549R, W1089X, and D1152H mutations.

We have developed a validated test using primer mix 2A for the 1717-1G>A, G542X, W1282X, N1303K, DF508 and 3849+10kbC>T mutations and have applied these in a thorough investigation of the incidence of the mutations in 100 individuals.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer in one or more cycles of PCR amplification. A convenient example of this aspect is set out in our European patent number EP-B1-0332435. In the present application, the further amplification primer is either a 1717-1G>A common, G542X common, W1282X common, N1303K common, DF508 common or 3849+10kbC>T common primer. The 1717-1G>A common primer TAATCTCTACCAAATCTGGATACTATACC is conveniently used in combination with the 1717-1G>A mutant primer, the G542X common primer TAATCTCTACCAAATCTGGATACTATACC is conveniently used in combination with the G542X mutant primer, the W1282X common primer GAATTCCCAAACTTTTAGAGACATC is conveniently used in combination with the W1282X mutant primer, the, N1303K common primer CTTGATGGTAAGTACATGGGTTTTTCTTAT is conveniently used in combination with the N1303K mutant primer, the DF508 common primer CCAGACTTCACTTCTAATTATGATTATGGG is conveniently used in combination with the DF508 mutant primer, the 3849+10kbC>T common primer TTGTGGATCAAATTTCAGTTGACTTGTCATC is conveniently used in combination with the 3849+10kbC>T mutant primer.

Any convenient control primer may be used. However, the amplification products from the Mix 1 primer mix serve as internal controls and so an additional amplification control is not required.

We have developed a validated test using primer mix 2B for the 621+1G>T, R553X, G551D, R117H, R1162X and R334W mutations and the normal DF508 allele and have applied these in a thorough investigation of the incidence of the mutations in 100 individuals.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer in one or more cycles of PCR amplification. A convenient example of this aspect is set out in our European patent number EP-B1-0332435. In the present application, the further amplification primer is either a 1717-1G>A common, G542X common, W1282X common, N1303K common, DF508 common or 3849+10kbC>T common primer. The DF508 common primer GACTTCACTTCTAATGATGATTATGGGAGA is

conveniently used in combination with the DF508 normal primer, the 621+1G>T common primer GTTTCACATAGTGTATGACCCTCTATATACACTCATT is conveniently used in combination with the 621+1G>T mutant primer, the R553X common primer ATCTAAAATTGGAGCAATGTTGTTTTTGACC is conveniently used in combination with the R553X mutant primer, the G551D common primer ATCTAAAATTGGAGCAATGTTGTTTTTGACC is conveniently used in combination with the G551D mutant primer, the R117H common primer GTTTCACATAGTGTATGACCCTCTATATACACTCATT is conveniently used in combination with the R117H mutant primer, the R1162X common primer TTTTGCTGTGAGATCTTTGACAGTCATTT is conveniently used in combination with the R1162X mutant primer, the R334W common primer TTTGTTTATTGCTCCAAGAGAGTCATACCA is conveniently used in combination with the R334W mutant primer.

Any convenient control primer may be used. We have selected control primers from unrelated regions of the genome, namely, part of the human apolipoprotein B gene and part of the ornithine decarboxylase gene.

We have developed a validated test using primer mix 2C for the A455E, 2183AA>G, 3659delC, 1078delT, DI507, R347P, S1251N and E60X mutations and have applied these in a thorough investigation of the incidence of the mutations in 100 individuals.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer in one or more cycles of PCR amplification. A convenient example of this aspect is set out in our European patent number EP-B1-0332435. In the present application, the further amplification primer is either a 621+1G>T common, R553X common, G551D common, R117H common, R1162X common, R334W common or A455E common primer. The A455E common primer GACTGACTGACTGACTGAAATGGAGACTTTTTGTTTATGTGGTTACTAA is conveniently used in combination with the A455E mutant primer, the 2183AA>G common primer GTATGATAGAGATTATATGCAATAAAACATTAACA is conveniently used in combination with the 2183AA>G mutant primer, the 3659delC common primer TGTGTCTAATATTGATTCTACTGTACAATAATAA is conveniently used in combination with the 3659delC mutant primer, the 1078delT common primer ATTTTTCCAAACTTCATTAGAACTGATCTATTGAC is conveniently used in

combination with the 1078delT mutant primer, the DI507 common primer CACAGTAGCTTACCCATAGAGGAAACA is conveniently used in combination with the DI507 mutant primer, the R347P common primer ATTTTTCCAAACTTCATTAGAACTGATCTATTGAC is conveniently used in combination with the R347P mutant primer, the S1251N common primer GCTCACCTGTGGTATCACTCCAA is conveniently used in combination with the S1251N mutant primer, the E60X common primer AATCAAACTATGTTAAGGGAAATAGGACAACTAA is conveniently used in combination with the E60X mutant primer.

Any convenient control primer may be used. We have selected control primers from unrelated regions of the genome, namely, part of the human apolipoprotein B gene and part of the ornithine decarboxylase gene.

We have developed a validated test using primer mix 3 for the G85E, 405+1G>A, S549R, W1089X, and D1152H mutations and have applied these in a thorough investigation of the incidence of the mutations in a number of individuals.

In many situations, it will be convenient to use a diagnostic primer of the invention with a further amplification primer in one or more cycles of PCR amplification. A convenient example of this aspect is set out in our European patent number EP-B1-0332435. In the present application, the further amplification primer is either a G85E common, 405+1G>A common, S549R common, W1089X common, D1152H common primer. The G85E and the 405+1G>A common primer CGATTCGATTCAGTTTTCTGTGGTTTCTTAGTGTTTGGA is conveniently used in combination with the G85E mutant primer and/or the 405+1G>A mutant primer, the S549R common primer GTAATTTTTTTACATGAATGACATTTACAGCAA is conveniently used in combination with the S549R mutant primer, the W1089X common primer GGAAATTATTTGTTTAACAATAAAACAATGGAA is conveniently used in combination with the W1089X mutant primer and the D1152H common primer CCAACAACACCTCCAATACCAGTAAC is conveniently used in combination with the D1152H mutant primer.

Any convenient control primer may be used. We have selected control primers from unrelated regions of the genome, namely, part of the human apolipoprotein B gene and part of the ornithine decarboxylase gene.

It will be appreciated that by combining intron 8 polyT allele-specific primers and their respective common primer and/or different mutant or normal primers and their respective common primers, or by using combinations of the 1 and 2A and/or 2B and/or 2C and/or 3 primer sets or mixes for a given DNA sample, further useful diagnostic tests are provided which permit the simultaneous detection of alternative polyT alleles and/or several mutations in the CFTR gene. Each of these combinations will also include the addition of the appropriate control primers as set out in Tables 1 to 5 below. The combination of different primers for the simultaneous detection of two or more point mutations is termed "multiplexing"(see EP-B1-0332435).

A variety of methods may be used to detect the presence or absence of diagnostic primer extension products and/or amplification products. These will be apparent to the person skilled in the art of nucleic acid detection procedures. Preferred methods avoid the need for radiolabelled reagents. Particular detection methods include size separation of amplification products, for example as described in our patents numbers EP 0 332 435 & US 5595890, "ALEX" product detection (Haque K. et al. Diag. Mol. Pathol. &: 248-252 (1998)), the detection of amplification incorporated "Sunrise" probes (Nazarenko IA. et al. Nucl. Acids Res. 25: 2516-2521 (1997)), the detection of amplification incorporated "Scorpions" primers (Whitcombe D. et al. Nature Biotechnol. 17: 804-807 (1999)), the detection of fluorescence polarisation signal combined with dual labelled ARMS primers (Gibson NJ. et al. Clin. Chem. 43: 1336-1341 (1997)) patent number EP 0 382 433, the detection of intercalating dyes into ARMS products (Brownie J. et al. Nucl. Acids Res. 25: 3235-3241 (1997)) patent number EP 0 731 177, "taqman" product detection for example as described in patent numbers US-A-5487972 & US-A-5210015; and "Molecular Beacons" product detection outlined in patent number WO-95/13399.

One or more of the primer mixes of the invention may be conveniently packaged with instructions for use in the method of the invention and appropriate packaging and sold as a kit. Convenient primer mixes include intron 8 polyT allele-specific primers and CFTR mutation-specific primers, equivalent normal-specific primers; all as hereinbefore disclosed. The kits will conveniently include one or more of the following: appropriate nucleotide triphosphates, for example one or more of dATP, dCTP, dGTP, and dTTP, a suitable polymerase as previously described, and a convenient buffer solution.

The invention will now be illustrated but not limited by reference to the following Examples and Figures in which:

Figure 1 shows the relative location of the PCR product bands on an agarose electrophoresis gel corresponding to (PolyT)

Figure 2 shows the relative location of the PCR product bands on an agarose electrophoresis gel corresponding to a CFTR DF508 heterozygote, DF508 homozygote, DF508/ DI507 compound heterozygote, G542X heterozygote, G551D heterozygote, 1078delT heterozygote. (i.e figure from brochure)

Figure 3 shows the relative location of the PCR product bands on an agarose electrophoresis gel corresponding to a (Middle Eastern Panel)

Figure 4 shows diagramatically the relative sizes in base pairs and the relative location of the PCR product bands on an agarose electrophoresis gel corresponding to the CFTR intron 8 5T, 7T and 9T alleles.

Figure 5 shows diagramatically the size in base pairs and the relative location of the PCR product bands on an agarose electrophoresis gel corresponding to the CFTR 1717-1G>A, G542X, W1282X, N1303K, DF508, 3849+10kbC>T, 621+1G>T, R553X, G551D, R117H, R1162X, R334W, A455E, 2183AA>G, 3659delC, 1078delT, DI507, R347P, S1251N, E6OX mutant alleles and the non-DF508 allele.

Figure 6 shows diagramatically the size in base pairs and the relative location of the PCR product bands on an agarose electrophoresis gel corresponding to the CFTR D1152H, W1089X, G85E, 405+1G>A, S549R mutant alleles.

Examples

Example 1.1 Materials Provided in a diagnostic kit containing *50*tests (polyT)

1. 50 Vial 1 (colour coded) containing primers (5T, 7T, 9T), and deoxynucleotide triphosphates in buffer.
2. 1 vial dilution buffer.
3. 1 vial AmpliTaq Gold.
4. 1 vial loading dye.
5. 1 vial DNA control, contains human DNA in buffer.
6. Instructions for use.

Example 2.1 Materials Provided in a diagnostic kit containing 50 tests (CF20)

1. 50 Vial 2A (colour coded) containing primers (mutant 1717-1G>A, G542X, W1282X, N1303K, DF508 and 3849+10kbC>T), control primers and deoxynucleotide triphosphates in buffer.
2. 50 Vial 2B (colour coded) containing primers (mutant 621+1G>T, R553X, G551D, R117H, R1162X, R334W, normal DF508), control primers and deoxynucleotide triphosphates in buffer.
3. 50 Vial 2C (colour coded) containing primers (mutant A455E, 2183AA>G, 3659delC, 1078delT, DI507, R347P, S1251N, E6OX), control primers and deoxynucleotide triphosphates in buffer.
4. 1 vial dilution buffer.
5. 1 vial AmpliTaq Gold.
6. 1 vial loading dye.
7. 1 vial normal DNA control, contains human DNA unaffected by the mutations detected by the kit, in buffer.
8. Instructions for use.

Example 3.1 Materials Provided in a diagnostic kit containing 50tests (Middle Eastern)

1. 50 Vial 3 (colour coded) containing primers (D1152H, W1089X, G85E, 405+1G>A, S549R), control primers and deoxynucleotide triphosphates in buffer
2. 1 vial dilution buffer.

3. 1 vial AmpliTaq Gold.

4. 1 vial loading dye.

5. 1 vial normal DNA control, contains human DNA unaffected by the mutations detected by the kit, in buffer.

6. Instructions for use.

Example 4.1 Instructions for CF20 PCR amplification procedure

1. Program the thermal cycler for an activation program which holds the vials at 94 °C for 20 minutes and an amplification program of 0.5 minutes at 94 °C (denaturation), 2 minutes at 58 °C (annealing) and 1 minute at 72 °C (extension) for 35 cycles. Followed by a 20 minute 72 °C extension stage..

2. Label one vial A, one vial B and one vial C for each sample and control.

3. Microfuge vials A, B and C until all liquid is at the bottom of each vial.

4. Prepare sufficient dilution of the AmpliTaq Gold for the number of samples and controls to be tested. For 10 samples or controls pipette 68 µL sterile deionized water, 20 µL dilution buffer, 100 µL loading dye and 12 µL AmpliTaq Gold into a microfuge tube and mix gently.

5. Carefully open the vial lid and pipette 5 µL of the enzyme dilution into the A, B and C vials using separate tips and re-cap.

6. Pipette 5 µL of test or Normal DNA Control sample to each of a vial A, B and C vials using separate tips. Add one drop of Sigma light white mineral oil to cover the aqueous phase if using a thermal cycler without a heated lid. Re-cap firmly.

7. For the negative control add 5 µL sterile water to a vial of each of A, B and C. Add one drop of Sigma light white mineral oil to cover the aqueous phase if using a thermal cycler without a heated lid. Re-cap firmly.

8. Microfuge vials A, B and C until all liquid is at the bottom of each vial.

9. Place all tubes firmly in the thermal cycler block. Initiate the 94 °C activation program. On completion of the activation program, run the amplification program.

10. On completion of the final extension stage , the samples may be stored at room temperature overnight or at 2-8 °C for up to 7 days before analysis by gel electrophoresis.

Instructions for CF Poly-T and CF-MEP PCR amplification procedure

1. Program the thermal cycler for an activation program which holds the vials at 94 °C for 20 minutes and an amplification program of 0.5 minutes at 94 °C (denaturation), 2 minutes at 58 °C (annealing) and 1 minute at 72 °C (extension) for 35 cycles. Followed by a 20 minute 72 °C extension stage..

2. Label a vial for each sample and control.

3. Microfuge vials until all liquid is at the bottom of each vial.

4. Prepare sufficient dilution of the AmpliTaq Gold for the number of samples and controls to be tested. For 10 samples or controls pipette 68 µL sterile deionized water, 20 µL dilution buffer, 100 µL loading dye and 12 µL AmpliTaq Gold into a microfuge tube and mix gently.

5. Carefully open the vial lid and pipette 5 µL of the enzyme dilution into the vials using separate tips and re-cap.

6. Pipette 5 µL of test or DNA Control sample to each vial using separate tips. Add one drop of Sigma light white mineral oil to cover the aqueous phase if using a thermal cycler without a heated lid. Re-cap firmly.

7. For the negative control add 5 µL sterile water to a separate vial . Add one drop of Sigma light white mineral oil to cover the aqueous phase if using a thermal cycler without a heated lid. Re-cap firmly.

8. Microfuge vials until all liquid is at the bottom of each vial.

9. Place all tubes firmly in the thermal cycler block. Initiate the 94 °C activation program. On completion of the activation program, run the amplification program.

10. On completion of the final extension stage, the samples may be stored at room temperature overnight or at 2-8 °C for up to 7 days before analysis by gel electrophoresis.

Example 4.2 Procedure

1. 15 x 12 cm horizontal submarine gels with combs of 1.5 mm x 5 mm suspended 1 mm above the gel tray, were prepared using 100 mL of 3% *NuSieve*™ (FMC Corporation) 3:1 agarose in 134 mM (16.2 g/L) Tris-base, 74.9 mM (4.63 g/L) boric acid, 2.55 mM (0.95 g/L) EDTA buffer with 0.1 µg/mL ethidium bromide (TBE/EtBr). TBE/EtBr was also used as the running buffer.

2. A 50 Base-Pair Ladder (Amersham Pharmacia Biotech) at 1.5 μg/15μL was prepared in the loading dye (80 μL distilled water / 10μL loading dye / 10μL 50 Base-Pair Ladder). 15μL of this dilution was run adjacent to samples as a molecular weight marker.

3. 15 μL of PCR products from samples or control vials were loaded on a gel (for CF20 A, B and C trios were loaded in adjacent wells).

4. Electrophoresis was carried out at 5 to 6 V/cm between electrodes until the dye front had migrated 5 cm from the loading wells towards the anode (1.5 to 2 hours).

5. After electrophoresis the gels were placed on a UV transilluminator at 260 nm then visualised and photographed.

Example 4.3 Interpretation of Results for CF20 and CF-MEP

PCR products will be observed as bands in the vial tracks of the gel.

1. The upper and lower control band must be clearly visible in all samples.

2. All tracks should be free of excessive smearing and background fluorescence.

3. The position of the upper and lower control bands should indicate the correct molecular size (see Figure 5 or Figure 6).

4. The negative control should show no bands in the tracks within the area corresponding to the upper and lower control bands. A diagnostic band should not be interpreted if a similar band is also seen in the negative control for that PCR run as this is indicative of contamination with genomic DNA.

If any of the above points are not observed the results should not be interpreted and a repeat test carried out.

5. An individual has two copies of the CFTR gene. Where these copies have the same sequence for any given site, an individual is described as being homozygous for this site. Where the copies differ in sequence at a given site, an individual is described as being heterozygous for this site.

6. The presence of PCR product generated from the normal DF508 primer in vial B of CF20 indicates that the sample contains the normal sequence for this site. The normal PCR product will be observed in the vial B track of the gel at 160bp and is identified by comparison of theband position with an adjacent marker track.

7. For CF20 the PCR products from an individual carrying any of the 1717-1G>A, G542X, W1282X, N1303K, DF508 and 3849+10kbC>T mutations will be observed in

the vial A track of the gel and are identified by comparison of the band position with an adjacent marker track. The product band sizes in base pairs are shown in Figure 5. Only product bands of the correct size should be interpreted.

8. For CF20 the PCR products from an individual carrying any of the 621+1G>T, R553X, G551D, R117H, R1162X, and R334W mutations will be observed in the vial B track of the gel and are identified by comparison of the band position with an adjacent marker track. The product band sizes in base pairs are shown in Figure 5. Only product bands of the correct size should be interpreted.

9. For CF20 the PCR products from an individual carrying any of the A455E, 2183AA>G, 3659delC, 1078delT, DI507, R347P, S1251N and E60X mutations will be observed in the vial C track of the gel and are identified by comparison of the band position with an adjacent marker track. The product band sizes in base pairs are shown in Figure 5. Only product bands of the correct size should be interpreted.

Interpretation of Results for CF Poly-T

PCR products will be observed as bands in the vial tracks of the gel.

1. The bands should be of similar intensity to the 250bp band in the 50bp ladder, loaded at 1.5mg/15ml (Amersham Pharmacia Biotech).

2. At least one of the CF Poly-T products should be visible in each DNA sample.

3. No bands should be visible in the negative control

4. If any of the above points are not observed the results should not be interpreted and a repeat test carried out.

5. For CF Poly-T the product band sizes in base pairs are shown in Figure 4. Only product bands of the correct size should be interpreted.

6. As a result of allele expansion within the microsatellite region $(TG)_{8-13}$, contiguous to the Poly-T region, the length of the diagnostic PCR products may vary. As a result very small product band shifts may be observed during electrophoresis. These shifts do not alter the interpretation of test results.

7. Owing to the nature of the sequence surrounding the Poly-T locus, heteroduplexes may occasionally be formed from the PCR products and may therefore be visible in some test sample tracks. The position of these heteroduplexes is indicated in figure

8. The observed heteroduplexes are summarised as follows:

A 7T/7T individual may have a heteroduplex band larger than the 7T allele but not as large as the 9T allele.

A 5T/7T individual may have a heteroduplex that runs in a similar position to the 9T allele. Therefore if all three diagnostic bands are visible the individual's genotype is 5T/7T. The heteroduplex, if present, is generally weaker than the corresponding 5T and 7T diagnostic products.

A 5T/9T individual occasionally has a heteroduplex which run larger than the 9T allele.

Owing to the sequence variability in the region proximal to the Poly-T repeat, additional (previously unseen) heteroduplexes may be observed.

Example 4.4 Performance Characteristics

For CF20 one hundred EDTA blood samples were tested using the procedures described in Examples 4.1 and 4.2 in a 'blind' in-house study. Samples were prepared using the method described in Example 4.5. Each result obtained was confirmed for each of the 20 mutations using alternative methods. Of the 100 individuals tested, 92 were normal and 8 were DF508 heterozygotes.

In addition blood and mouthwash samples were taken from each of 40 individuals and tested using the diagnostic assay. The result obtained from each mouthwash sample was concordant with that obtained using the blood sample from the same individual.

A number of compound heterozygotes within the same multiplex test were sourced and analysed using the diagnostic assay. In all cases, both mutations were detected. Those tested were:

The 'A mix'

1717-1G>A / N1303K, 1717-1G>A / DF508, G542X / W1282X, G542X / N1303K, G542X / DF508, W1282X / N1303K, W1282X / DF508 , W1282X /3849+10kbC>T, N1303K / DF508, DF508 / 3849+10kbC>T.

The 'B mix'

R553X / G551D, R553X / R334W.

Owing to the rarity of the remaining combinations and the subsequent unavailability of samples, other compound heterozygotes were not evaluated.

The rare R1283M, 1717-2A>G, R117C, 621+2T>C, R117L, I506V mutations have been evaluated for cross reactivity. These were tested using the procedures described in Examples 1.2 and 1.3 and were not detected. In addition the following polymorphisms were not detected by the test: 3617G/T, 1655T/G (F508C), 1651A/G.

Evaluation of known mutations and polymorphisms in the CFTR gene using the procedures described in Examples 4.1 and 4.2 has highlighted the following observations:

1. DI507 in combination with dupl 716+51 >61 will produce a PCR product of 233bp in the 'C mix' rather than the expected 222bp.

2. Slight cross reactivity of the R347P primer in the 'C mix' with the rare R347H mutation was observed which results in faint PCR product visible at the R347P position in the 'C mix'.

3. A recently reported mutation, 2184insG , will theoretically cross react with the 2183AA>G primer in the 'C mix'. A diagnostic 2183AA>G band may indicate that the 2184insG mutation is present.

4. The following mutations, which have not been checked due to unavailability of relevant samples, may interfere with test function: R117P, 621+2T>G, R553G, R553Q, R347L, I506T, I506S and the rare combination of DI507 with the polymorphism 1651A/G. For CF Poly-T one hundred EDTA blood samples were tested using the procedures described in Examples 4.1 and 4.2 in a 'blind' in-house study. Samples were prepared using the method described in Example 4.5. The genotype for each sample was independently confirmed. Additionally 100 mouthwash samples paired with the blood samples were tested on the CF Poly-T test. The result obtained from each mouthwash sample was concordant with that obtained using the blood sample from the same individual.

There are a number of sequence variants in the CFTR gene of unknown frequency which are located close to the Poly-T tract. The following mutations, which have not been checked due to unavailability of relevant samples, may theorectically interfere with test function:

1. The 1342-11TTT>G mutation which results in a $(TG)_{13}(T)_3$ individual with an apparent 5T disease state will produce diagnostic PCR product from the 5T primer.

2. The 1342-13G/T polymorphism which alters the final TG repeat to TT by substitution of the final does not affect the performance of the test as this polymorphism changes the status of individuals with respect to the polythymidine tract length.

3. The effects of the 1342-1G>C, and 1342-2A>C and 1342-2delAG mutations have not been tested but may affect the performance of the ELUCIGENE™ CF Poly-T test.

For CF-MEP one hundred EDTA blood samples were tested using the procedures described in Examples 4.1 and 4.2 in a 'blind' in-house study. Samples were prepared using the method described in Example 4.5. All 100 samples tested were normal with respect to the CF-MEP mutations.

One compound heterozygotes within the same multiplex test was sourced and analysed using the diagnostic assay. In the sample G85E/D1152H both mutations were detected

Example 4.5 Method for Preparation of DNA from Whole Blood (EDTA) Samples:

1. Pipette 80 µL of each blood sample into a screw-topped microfuge tube.
2. Pipette 320 µL of 170 mM (9.09 g/L) NH_4Cl solution into each tube.
3. Mix for 20 minutes by gentle swirling and inversion. Avoid vigorous agitation and formation of foam.
4. Centrifuge each tube for 2 minutes at 12 000g until a cell pellet is formed.
5. Using a pipette remove and discard the supernatant liquid.
6. Pipette 300 µL of 10 mM (0.58 g/L) NaCl/10 mM (3.72 g/L) EDTA into each tube and resuspend the cells by vortex mixing.
7. Centrifuge each tube for 1 minute at 12 000g until a cell pellet is formed.
8. Repeat steps 5 to 7 at least a further two times until all visible red coloration in the supernatant liquid has been removed.
9. Using a pipette remove and discard the supernatant liquid.
10. Pipette 200 µL of 50 mM (2 g/L) NaOH solution into each tube and resuspend the cells by vortex mixing.
11. Incubate at 100°C for 10 minutes.
12. Pipette 40 µL of 1 M (121.1 g/L) Tris/HCl (pH 7.5) into each tube and vortex mix.

13. Add 1ml sterile deionised water to each microfuge tube to give a total DNA sample volume of 1.24mL.

14. Centrifuge each tube for 1 minute at 12 000g until a pellet of cell debris is formed. The DNA is contained within the supernatant liquid.

Example 4.6 Method for Preparation of DNA from Mouthwash Samples:

1. Agitate 10 mL of 0.9% saline in the mouth for 20 seconds. Collect the suspension in a sterile plastic universal tube.

2. Pellet the cells by centrifugation at 800g for 10 minutes at 18-28 °C.

3. Using a pipette remove and discard the supernatant liquid.

4. Pipette 500 µL of 10 mM (0.58 g/L) NaCl/10 mM (3.72 g/L) EDTA into each tube and resuspend the cells by vortex mixing.

5. Transfer each sample to a screw-capped microfuge tube.

6. Centrifuge each tube for 1 minute at 12 000g until a cell pellet is formed.

7. Using a pipette carefully remove and discard the supernatant liquid.

8. Pipette 500 µL of 50 mM (2g/L) NaOH solution into each tube and resuspend the cells by vortex mixing.

9. Incubate at 100°C for 10 minutes

10. Pipette 100 µL of 1 M (121.1 g/L) Tris/HCl (pH 7.5) into each tube and vortex mix.

11. Centrifuge each tube for 1 minute at 12 000g until a pellet of cell debris is formed. The DNA is contained within the supernatant liquid.

12. Transfer 100 µL of the supernatant (DNA sample) to a fresh, labelled microfuge tube.

13. Add 400 µL of sterile deionised water to each DNA sample to give a total volume of 500 µL.

The mix formulation for mix 1 is presented below (Table 1). The **25**µl total reaction volume also includes 1x ARMS, 100mM dNTP, 1.5U AmpliTaq Gold, 1x CRS.

Table 1

Primer	F/R	Primer Ref	Mismatch	Length	Sequence
5T	R	5TMR	GT-3	26	TAA TTC CCC AAA TCC CTG TTA AAG AC
7T	R	7T25	GT-2	53	GTT AAT CAT TCA GCT ACT ACG CAC CTA ATT CCC CAA ATC CCT GTT AAA AAA GC
9T	R	9T60	TT-2	90	GAC TGT ACG ATA CTC ATT TAT ATG AAG TCA GCT TAC TTA CCT ATA GAA CGC TTG CTA GTT TAA TTC CCC AAA TCC CTG TTA AAA AAA ATC
Intron 8 polyT C	F	PT COM FOR		35	GTA CAT AAA ACA AGC ATC TAT TGA AAA TAT CTG AC

The mix formulation for the 2A mix is presented below (Table 2). The 25µl total reaction volume also includes 1x ARMS, 100mM dNTP, 1.5U AmpliTaq Gold, 1x CRS

Table 2

	F/R	Primer Ref	Mismatch	Length	Conc µM	Sequence
Apo B	F	ABF	CA-2	23	0.2	GAG CAC AGT ACG AAA AAC CAC CT
Apo B	R	ABR20A	TG-2 AG-6	25	0.2	AAA CAC GAA GAT GCT GTC TAC TAT C
ODC (FPLC purified)	F	ODFFPLC	CT-2	30	0.3	AGA GGA TTA TCT ATG CAA ATC CTT GTA ACC
ODC (FPLC purified)	R	ODRFPLC	AC-2	30	0.3	TCA ACT TCA CTA TCA AAA GTC ATC ATC TAG
W1282X M	F	WXMF	CC-2	30	1	TCT TGG GAT TCA ATA ACT TTG CAA CAG TCA
W1282X C	R	WXCR		25	1	GAA TTC CCA AAC TTT TAG AGA CAT C
1717-1G>A M	F	17MF20	TT-2 GA-23	40	2	TAC TAA AAG TGA CTC TCG AAT TTT CTA TTT TTG GTA ATT A
Exon 11 C	R	Aex11CR		29	1	TAA TCT CTA CCA AAT CTG GAT ACT ATA CC
G542X M	F	GXMF	CA-2	30	1	AGT TTG CAG AGA AAG ACA ATA TAG TTC TCT
N1303K C	F	NKCF2	TC-9	30	1	CTT GAT GGT AAG TAC

		0				ATG GGT TTT TCT TAT
N1303K M	R	NKMR	TT-2	30	1	TGA TCA CTC CAC TGT TCA TAG GGA TCC ATC
DF508 C	F	ADFCF 20	TC-12	30	0.5	CCA GAC TTC ACT TCT AAT TAT GAT TAT GGG
DF508 M	R	ADFMR	TT-2	30	0.5	GTA TCT ATA TTC ATC ATA GGA AAC ACC ATT
3849+10kb C	F	38CF		31	0.2	TTG TGG ATC AAA TTT CAG TTG ACT TGT CAT C
3849+10kb M	R	38MR20	GA-3	29	0.2	GAA CAT TTC CTT TCA GGG TGT CTT ACG CA

The mix formulation for the 2B mix is presented below (Table 3). The 25µl total reaction volume also includes 1x ARMS, 100mM dNTP, 1.5U AmpliTaq Gold, 1x CRS.

Table 3

Primer	**F/ R**	**Primer Ref**	**Mismatch**	**Length**	**Conc µM**	**Sequence**
Apo B	F	ABF	CA-2	23	0.1	GAG CAC AGT ACG AAA AAC CAC CT
Apo B	R	ABR20B	CA-2	26	0.1	CAT TTA GTT TCA GCC CAG GAA TAA CG
ODC (FPLC purified)	F	ODFFPLC	CT-2	30	0.3	AGA GGA TTA TCT ATG CAA ATC CTT GTA ACC
ODC (FPLC purified)	R	ODRFPLC	AC-2	30	0.3	TCA ACT TCA CTA TCA AAA GTC ATC ATC TAG

621+1G>T M	R	62MR	GT-2	30	2	TGC CAT GGG GCC TGT GCA AGG AAG TAT TGA
R117H M	R	RHMR	CC-2	30	0.5	AGC CTA TGC CTA GAT AAA TCG CGA TAG ACT
621/R117H C	F	ex4CF	CT-8 TC-26 GA-27	37	1.5	GTT TCA CAT AGT GTA TGA CCC TCT ATA TAC ACT CAT T
R334W M	F	RWMF	GG-2	30	0.2	CCT ATG CAC TAA TCA AAG GAA TCA TCC TGT
R334W C	R	RWCR		30	0.2	TTT GTT TAT TGC TCC AAG AGA GTC ATA CCA
G551D M	R	GDMR20	TT-2	27	2	TTG CTA AAG AAA TTC TTG CTC GTT GTT
R553X M	R	R5MR	GG-2	60	2	GAC TGA CTG ACT GAC TGA CTC TGA CTG ACT TAT TCA CCT TGC TAA AGA AAT TCT TGC TGA
G551D/ R553X C	F	Bex11CF20	GG-20 GA-21	31	2	ATC TAA AAT TGG AGC AAT GTT GTT TTT GAC C
DF508 N	R	BDFNR	CT-2	30	0.5	GTA TCT ATA TTC ATC ATA GGA AAC ACC ACA
DF508 C	F	BDFCF		30	0.5	GAC TTC ACT TCT AAT GAT GAT TAT GGG AGA
R1162X M	F	R1MF	TG-2	30	0.2	TAT TTT TAT TTC AGA TGC GAT CTG TGA GTT
R1162X C	R	R1CR		29	0.2	TTT TGC TGT GAG ATC TTT GAC AGT CAT TT

The mix formulation for the 2C mix is presented below (Table 4). The 25µl total reaction volume also includes 1x ARMS, 100mM dNTP, 1.5U AmpliTaq Gold, 1x CRS.

Table 4

Primer	F/R	Primer Ref	Mismatch	Length	Conc µM	Sequence
Apo B	F	ABF	CA-2	23	0.2	GAG CAC AGT ACG AAA AAC CAC CT
Apo B	R	ABR20C		30	0.2	CAG CTT CTT ATA GAT TTG TAT TTC TCT GAA
ODC (FPLC purified)	F	ODFFPLC	CT-2	30	0.3	AGA GGA TTA TCT ATG CAA ATC CTT GTA ACC
ODC (FPLC purified)	R	ODRCFPLC	AC-2	31	0.3	TCA ACT TCA CTA TCA AAA GTC ATC ATC TGA A
A455E M	F	A45MF	TC-2	50	1	GAC TGA CTG ACT GAC TGA AAT TTC AAG ATA GAA AGA GGA CAG TTG TTG TA
A455E C	R	A45CR		49	1	GAC TGA CTG ACT GAC TGA AAT GGA GAC TTT TTG TTT ATG TGG TTA CTA A
2183AA>GC	F	2183CF		35	2	GTA TGA TAG AGA TTA TAT GCA ATA AAA CAT TAA CA
2183AA>GM	R	2183MR	AA-3	31	2	CCC AAA CTC TCC AGT CTG TTT AAA AGA TAG C
3659delCM	F	3659MF	GG-2	27	2	GAC ATG CCA ACA GAA GGT AAA CCT AGA
3659delC C	R	3659CR	CA-29	34	2	TGT GTC TAA TAT TGA TTC

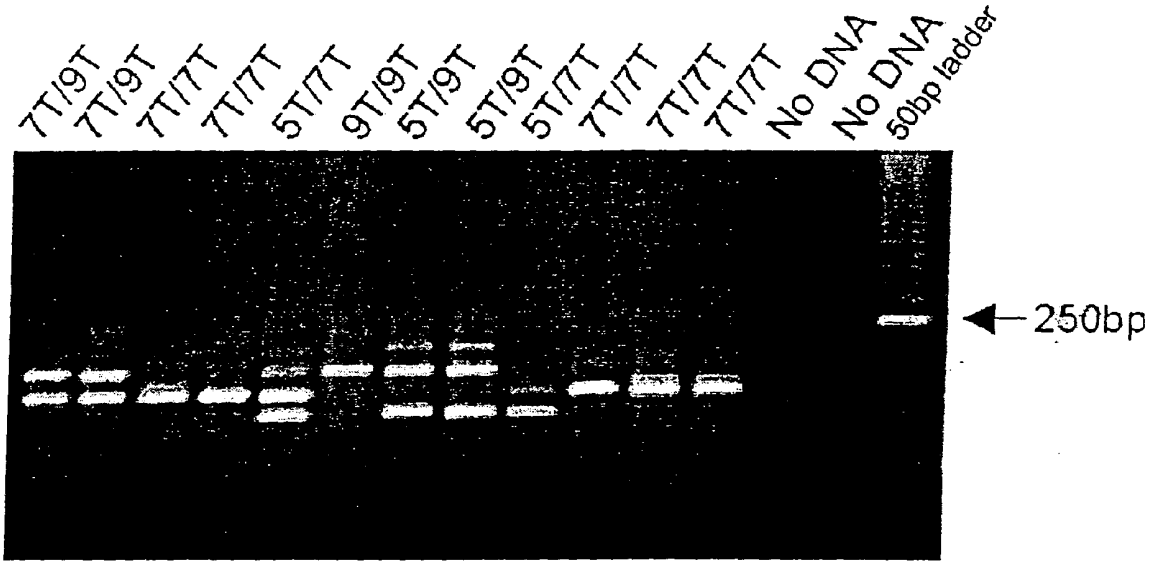
						TAC TGT ACA ATA ATA A
DI507 M	F	DIMF	AA-3	26	0.5	GCC TGG CAC CAT TAA AC AAA TAA CT
DI507 C	R	DICR		27	0.5	CAC AGT AGC TTA CCC ATA GAG GAA ACA
1078delT M	F	1078MF	CA-3	23	2	CCT TCT TCT TCT CAG GGT TCC TG
R347P M	F	R34PMF	AC-3	27	0.2	CAC CAT CTC ATT CTG CAT TGT TCT ACC
Exon 7 C	R	ex7CR	CC-24 CC-29	35	1	ATT TTT CCA AAC TTC ATT AGA ACT GAT CTA TTG AC
S1251N M	F	S12MF	CT-2	23	0.2	GGA AGA ACT GGA TCA GGG AAG CA
S1251N C	R	S12CR		23	0.2	GCT CAC CTG TGG TAT CAC TCC AA
E60X C	F	E60XCF		34	0.5	AAT CAA ACT ATG TTA AGG GAA ATA GGA CAA CTA A
E60X M	R	E60XMR	TG-3	26	0.5	TTA GGA TTT TTC TTT GAA GCC AGT TA

The mix formulation for mix 3 is presented below (Table 5). The 25µl total reaction volume also includes 1x ARMS, 100mM dNTP, 1.5U AmpliTaq Gold, 1x CRS.

Table 5

Primer	F/R	Primer Ref	Mismatch	Length	Conc µM	Sequence
Apo B	F	ABF	CA-2	23	0.1	GAG CAC AGT ACG AAA AAC CAC CT
Apo B	R	ABR20B	CA-2	26	0.1	CAT TTA GTT TCA GCC CAG GAA TAA CG
ODC (FPLC purified)	F	ODFFPLC	CT-2	30	0.3	AGA GGA TTA TCT ATG CAA ATC CTT GTA ACC
ODC (FPLC purified)	R	ODRCFPLC	AC-2	31	0.3	TCA ACT TCA CTA TCA AAA GTC ATC ATC TGA A
S549R C	R	122-99		33	0.5	GTA ATT TTT TTA CAT GAA TGA CAT TTA CAG CAA
S549R T>G M	F	126-99	CC-2	25	0.5	TGG AGA AGG TGG AAT CAC ACT GAC G
W1089X C	R	F10682		33	1.0	GGA AAT TAT TTG TTT AAC AAT AAA ACA ATG GAA
W1089X M	F	140-99	AG-3	28	1.0	AAG CTC TGA ATT TAC ATA CTG CCA AAT A
D1152H C	F	F10683		26	0.5	CCA ACA ACA CCT CCA ATA CCA GTA AC
D1152H M	R	145-99	TT-3	33	0.5	AAA GAT GAT AAG ACT

						TAC CAA GCT ATC CAC TTG
exon3 C (NH-tail)	R	670-99	TC-21. CT-22	39	0.5	CGA TTC GAT TCA GTT TTC TGT GGT TTC TTA GTG TTT GGA
G85E M (NH-tail)	F	616-99	CA-3	50	0.5	TAG CCA TTG ATG ACG GAG CGA TGT TTT TTC TGG AGA TTT ATG TTC TAC GA
405+1G>A M	F	134-99	TC-3	36	1.0	GAT TTA TGT TCT ATG GAA TCT TTT TAT ATT TAG TGA



Typical Results from the CF *Poly-T* test

FIGURE 1

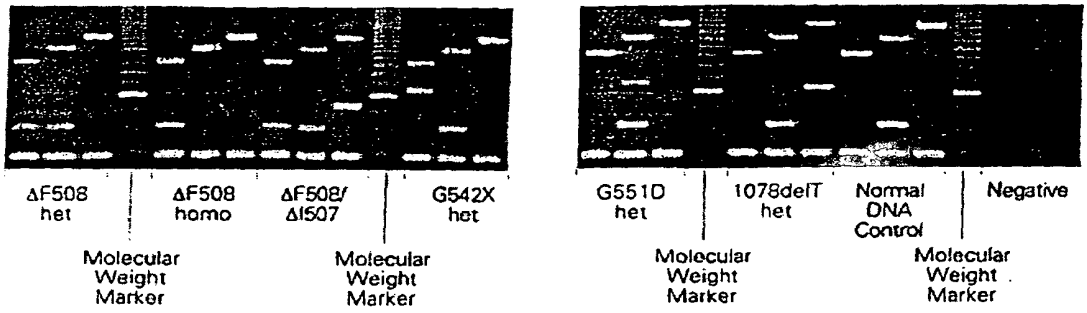


FIGURE 2

CF-MEP AGAROSE GEL RESULTS

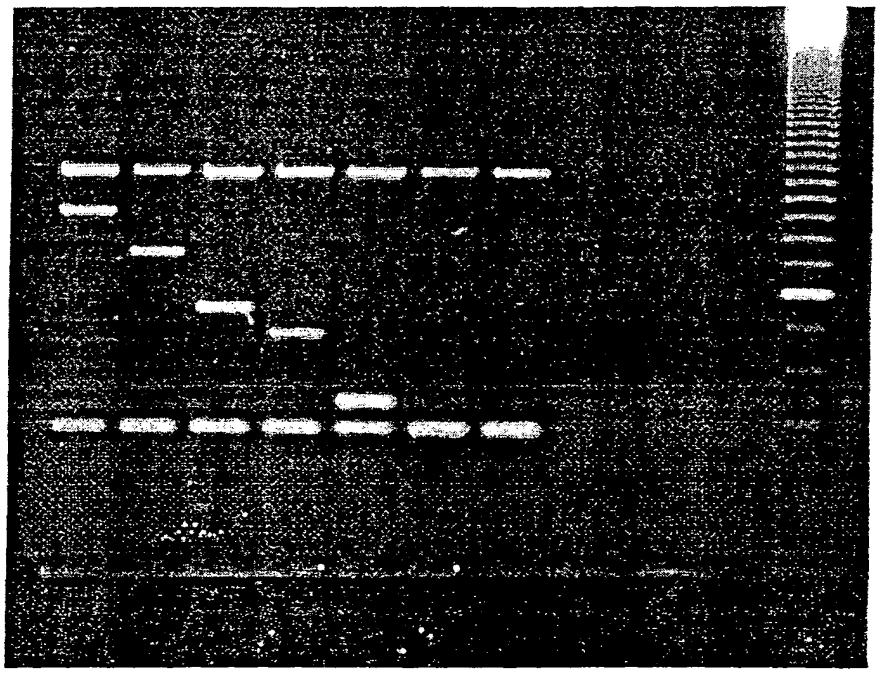


FIGURE 3

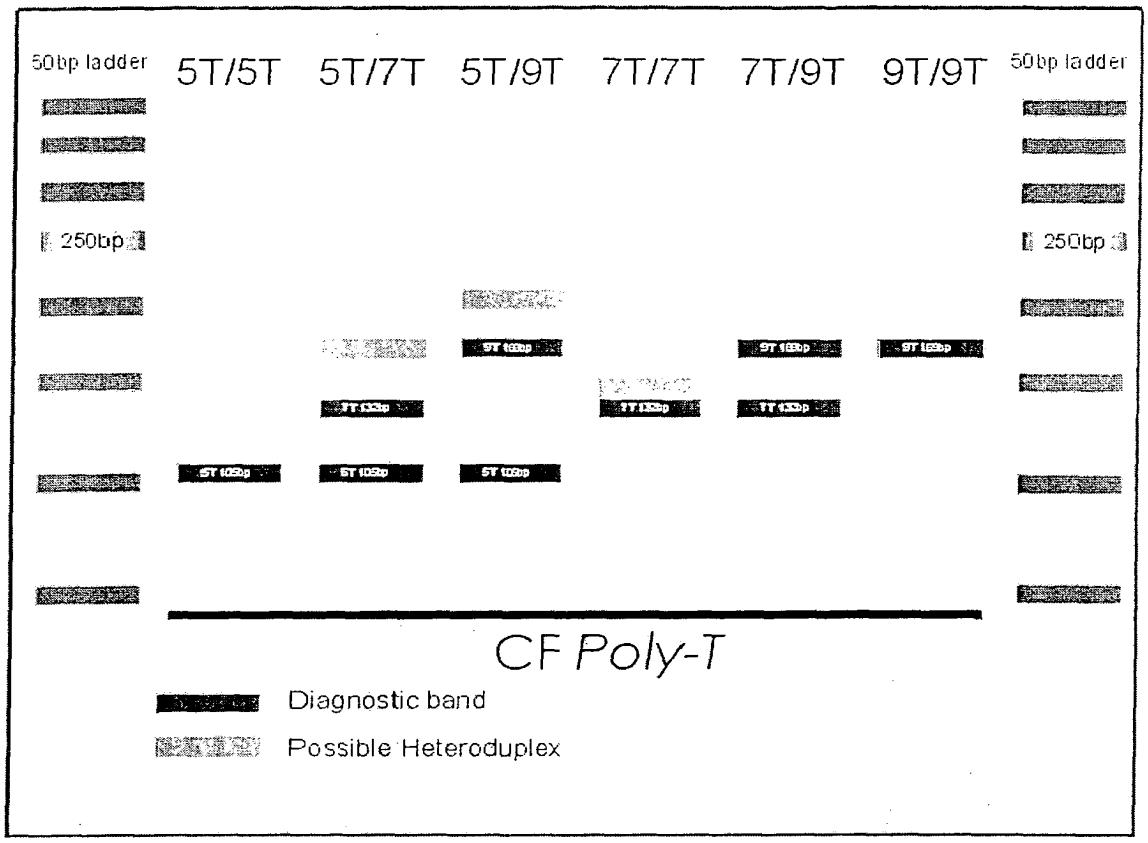
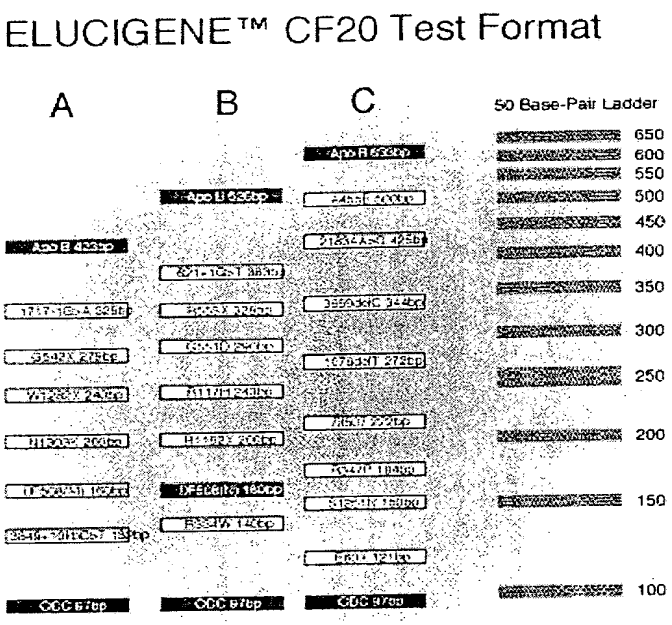

50bp ladder
5T/5T
5T/7T
5T/9T
7T/7T
7T/9T
9T/9T
50bp ladder
250bp
250bp
CF Poly-T
Diagnostic band
Possible Heteroduplex

FIGURE 4


ELUCIGENE™ CF20 Test Format
A
B
C
50 Base-Pair Ladder
650
600
550
500
450
400
350
300
250
200
150
100

5

FIGURE 5

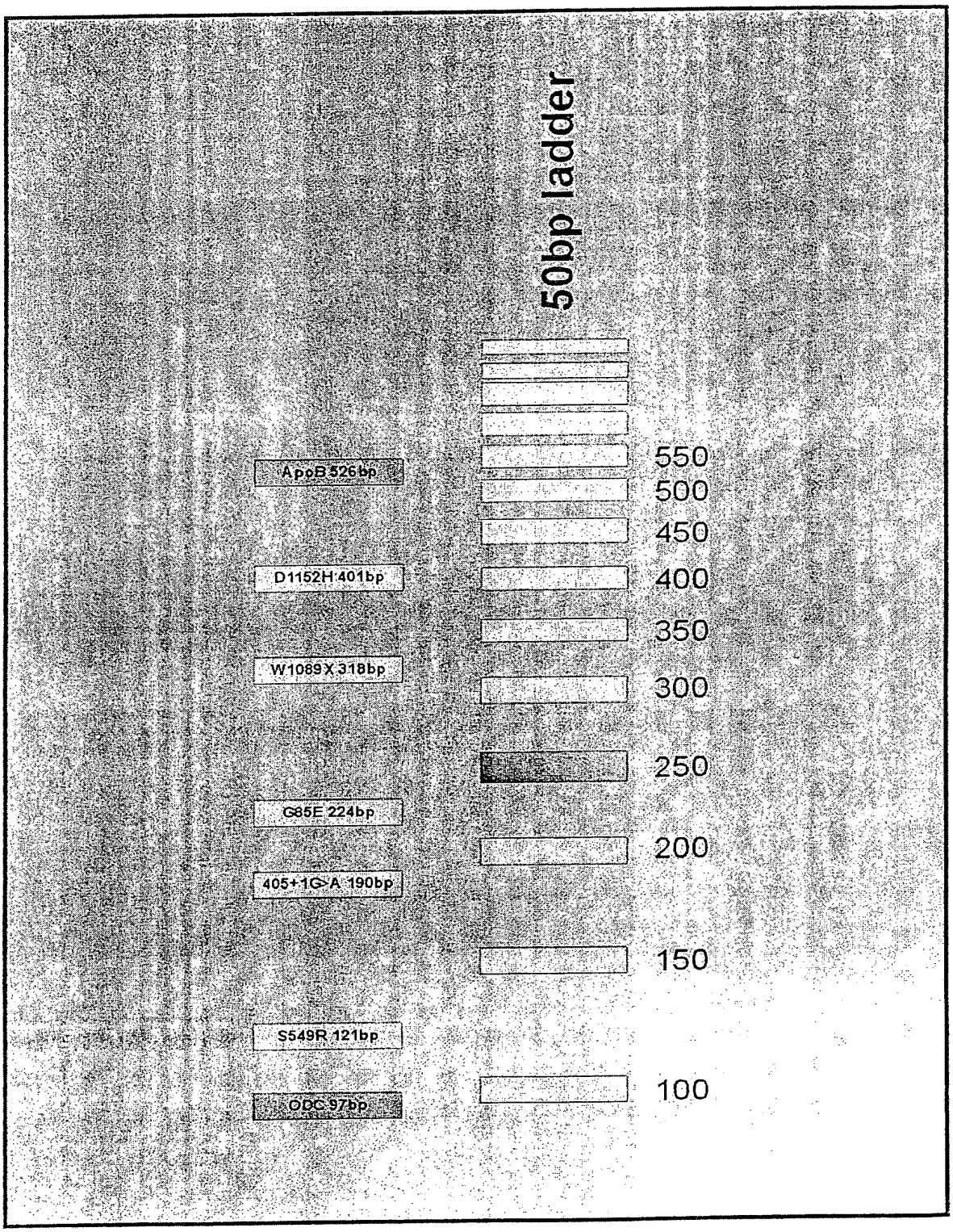


FIGURE 6

CT 68500.0-79

(Fraser Dawiss)